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Studies of the Specificity and Cross-Reactions of Antibodies to Lipid A Found in Juvenile Arthritis

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This work was started to determine whether the immunoglobulin G (IgG) reactions with monophosphoryl lipid A (MPL) found in children with arthritis were due to contaminants, a specific site on lipid A, or polyspecific binding. Different lots of MPL were examined by electrophoresis and immunoblot. Competitive inhibition of enzyme-linked immunosorbent assays (ELISAs) by analogs of MPL and biologic materials of clinical interest was used to determine the specificity of the binding site and potential cross-reactions. IgG in all patient sera tested reacted with a single band just <6.5 kDa on immunoblots of all lots of MPL tested. The ELISAs were inhibited best by analogs of lipid A with an exposed diglucosamine core and intact polar domains. The anti-MPL was also inhibited by fetal bovine collagen types I and II and in some instances by cardiolipin, but not by keratan sulfate, proteoglycan, or DnaK heat shock protein. Lot variation was a persistent technical problem, but no protein contaminant could be demonstrated in any lot. The ELISA and immunoblot results confirmed each other. Immunoblots detected a single band of MPL reactive with IgG. This antibody remains of interest because of its disease association and correlations and because it cross-reacts with collagen and cardiolipin.

This laboratory has been pursuing the discovery that naturally occurring immunoglobulin G (IgG) anti-monophosphoryl lipid A (MPL) antibody levels are elevated in sera from children with pauciarticular and polyarticular arthritis compared with normal children, children with systemic-onset arthritis, children with systemic lupus erythematosus, and children with chronic *Pseudomonas* infections secondary to cystic fibrosis (10, 12). More importantly, the titer of anti-MPL IgG correlates with the concentration of C3a and the C3d/C3 ratio of plasma in children with pauciarticular arthritis (12), and the concentrations of anti-MPL IgG and IgM are higher in synovial fluids from children with pauci- or polyarticular arthritis than in serum (10). The levels of antibodies to the mutant R595 lipopolysaccharide (2-keto-3-deoxyoctonate [KDO]-lipid A) from which the MPL was derived were also elevated but did not correlate with complement activation product concentration. KDO-lipid A only slightly inhibited the anti-MPL reaction (12). The presence of anti-MPL IgG in high titer in children is not unique to arthritis (9) but is unusual in its constancy, particularly for the association with complement activation.

Dealing with MPL and other forms of lipid A as antigens in water-based assays is difficult. MPL is largely hydrophobic, consisting of four to seven fully saturated fatty acid chains that vary in length from 10 to 18 carbons. The four primary fatty acids are attached to a diglucosamine core, the only polar region of the molecule except for carboxyl groups at carbon 3 of the fatty acids. In the buffers used for immunologic work, the molecules exist as oligomers (5), micelles, and bilayers (14). The factors which control the physical nature of the molecule in buffers are not understood but are known to be complex (5, 14). The epitopes reactive with induced antibodies in mice and rabbits are small segments of the diglucosamine core and the attached phosphoryl groups (8).

In our experience, different batches of MPL gave different absolute values with the same sera, although titers were correlated from batch to batch. One batch of MPL that we obtained had absolutely no reaction in the enzyme-linked immunosorbent assay (ELISA) at all, while another reacted with IgG in all sera. Therefore, it remained possible that the anti-MPL reaction in our studies involved a contaminant derived from the bacterial source of the MPL.

The studies reported here were initiated to determine (i) whether we could demonstrate a contaminant which was interacting with the sera of our patients, (ii) which region of the MPL molecule the IgG was reacting with, and (iii) whether there were cross-reactions between anti-MPL and other antigens of potential clinical significance, as has been reported for the IgM monoclonal antibodies developed for therapeutic use (1).

MATERIALS AND METHODS

Subjects and samples. Serum and synovial fluid samples were obtained from children attending the Rheumatic Disease Clinic at Children's Hospital at Stanford with the consent of the child and/or the parent. Blood and synovial fluid were collected only at times of clinical necessity. All patients studied met the criteria of the American Rheumatism Association for pauciarticular or polyarticular arthritis. Children known to have HLA-B27 antigen or a clinical diagnosis of spondyloarthropathy were excluded from the studies reported here because too few were available for study. None of the children had rheumatoid factor by hemagglutination (Rheumaton; Wampole Laboratories). Although our earlier studies used control sera from children, of which 10 to 20% may have positive ELISA titers by these methods, these are hard to obtain, and sera which were negative by the ELISA but obtained from normal adult laboratory workers were used for controls in this particular study. All samples, both serum and synovial fluid, were centrifuged to remove cells within 1 to 2 h of collection and stored at −70°C.

Lipid A preparations. Lipopolysaccharide (LPS) from the mutant strain of *Salmonella minnesota* R595 was obtained from List Biological Laboratories (Campbell, Calif.). This LPS lacks the polysaccharide normally associated with wild-type isolates and consists solely of a core oliogosaccharide containing two 2-keto-3-deoxyoctonate (KDO) molecules and diphosphoryl lipid A, and we refer to it as KDO-lipid A. MPL, also derived from *S. minnesota* R595, was obtained from List (lots 14, 15, and 24A) and from Ribi ImmunoChem Research, Inc. (Hamilton, Mont.) (lots 171, 181, and 201). Alkali-treated MPL was prepared by the method of Galanos et al. (6). This treatment putatively removes the esterified fatty acid groups but leaves the *N*-acetyl-D-glucosamine backbone intact (6). Synthetic MPLs, LA-16-PH and LA-15-PH, were purchased from ICN

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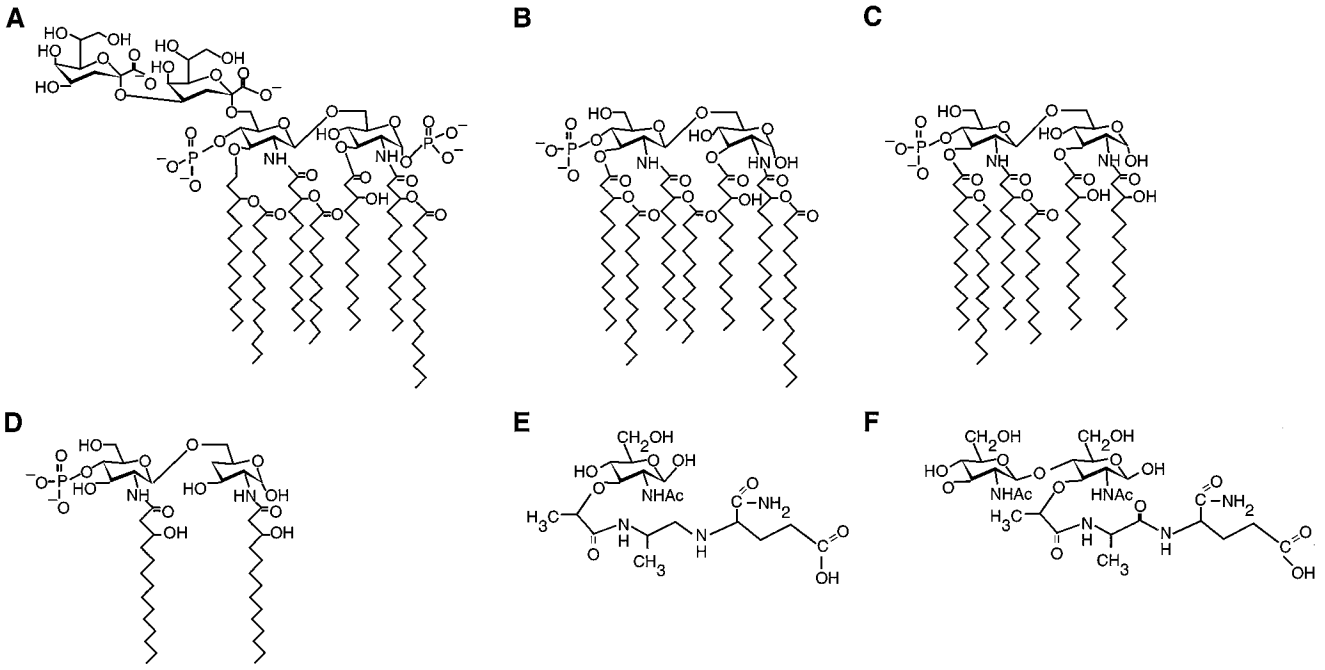

FIG. 1. Schematic drawings showing the structural differences in the MPL analogs used for inhibition studies of the epitope responsible for the anti-MPL reaction in children with arthritis. (A) KDO-lipid A (*S. minnesota* R595 LPS); (B) MPL and synthetic MPL-A LA-16-PH; (C) synthetic MPL LA-15-PH; (D) putative structure of alkali-treated MPL; (E) MDP; (F) GMDP.

Biomedicals, Inc. (Cleveland, Ohio). All products were prepared according to the manufacturer's specifications using either 0.2 or 0.5% triethylamine and sonication. Muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine [MDP]) and glucosaminyl muramyl peptides [*N*-acetyl-D-glucosaminyl-(1-4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (GMDP)] were purchased from Chemicon International (Temecula, Calif.). The structures of these various analogs of the MPL are shown in Fig. 1.

Other antigens. Cardiolipin from bovine heart was purchased from Sigma Chemical Company (St. Louis, Mo.). Proteoglycan and fetal cartilage collagen types I and II were kindly provided by Lane Smith, Department of Orthopedics, Stanford University School of Medicine, Stanford, Calif. DnaK protein, a 70-kDa heat shock protein from *Escherichia coli*, was purchased from Epicentre Technologies (Madison, Wis.).

Immunoblot analysis. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed by methods similar to those of Pollack et al. (13). MPL or KDO-lipid A samples were mixed with an equal volume of 0.1 M Tris-HCl buffer (pH 6.8) containing 2% sodium deodecyl sulfate (SDS), 20% sucrose, and 1% 2-mercaptoethanol and heated at 95°C for 5 min. Initially the samples were run in a Laemmli SDS-PAGE system with a 4% stacking gel and a 12.5% separating gel incorporating 4 M urea. Later, gels of 15% were used for better separation of the lower-molecular-weight components. No SDS was used in either the stacking or separating gel, as outlined by Hitchcock and Brown (7). The gel was electrophoresed at 30 mA per gel until the pyronin Y dye in the sample buffer had migrated 10 cm. Gels to be silver stained were fixed overnight in 40% ethanol–7% acetic acid. Rapid-Ag-Stain (ICN Radiochemicals, Irvine, Calif.) was used according to the manufacturer's directions. Protein molecular weight standards were used for reference because no phospholipid standards were available.

MPL or KDO-lipid A separated on identical, unstained companion gels was transferred to either 0.2-μm nitrocellulose or 0.2-μm polyvinylidene difluoride membranes (Bio-Rad Laboratories, Richmond, Calif.) by the method of Towbin et al. (18). Transfers were made in the cold at 70 V for 30 min.

Immunoblots were developed as follows. The membranes were immersed with gentle shaking for 60 min in a blocking solution of Tris-buffered saline, pH 7.5 (TBS), plus 2% bovine serum albumin (BSA) (Sigma). The membranes were washed three times in TBS with 0.05% Tween 20 and then incubated for 3 h in test serum diluted 1:20 in TBS–2% BSA–0.05% Tween 20. The membranes were washed again as above before the addition of horseradish peroxidase-labeled anti-immunoglobulin diluted in TBS–2% BSA–0.05% Tween. Incubation was continued with gentle shaking for 60 min. The membranes were washed, and color was developed by using diaminobenzidine with $NiCl_2$ enhancement.

ELISA for anti-lipid A. Serum and synovial fluid samples were tested for IgG and IgM antibodies to MPL by an adaptation of the method described previously (10, 12) and similar to those used by others (1, 8, 14). Briefly, Polysorb immunoplate wells (Nunc, Roskilde, Denmark) were coated overnight at 4°C with a 50-μg/ml suspension of MPL in a 0.05 M HCO_3 buffer, pH 9.6, and then washed five times with phosphate-buffered saline (PBS) containing 0.1 mg of $CaCl_2$ and 0.1 mg of $MgCl_2$ per ml plus 0.05% Tween 20. Centrifuged samples were serially diluted from 1:10 with the same PBS to which 1% BSA had been added. The same batch of BSA was used throughout, avoiding possible effects of variation in LPS contamination. Diluted samples were incubated in the coated plates for 1 h at room temperature, washed five times with buffer, and reincubated for 1 h at room temperature with goat anti-human IgG or anti-IgM coupled to peroxidase

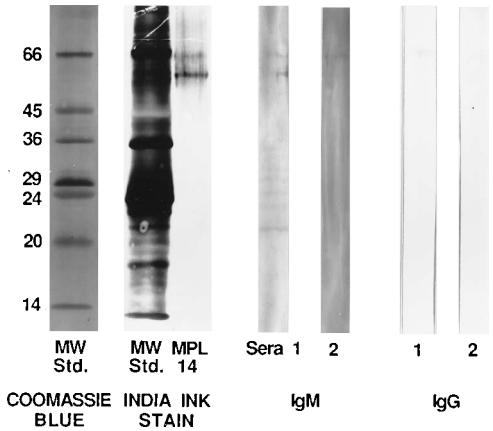

FIG. 2. Nitrocellulose after transfer from the 12.5% polyacrylamide gel, showing the different patterns of staining of the molecular weight standards (MW Std.) with Coomassie blue and India ink, and the India ink stain of MPL List lot 14. The latter shows transfer of bands at approximately 60 and 66 kDa. The four right-hand channels show immunoblot results with two different ELISA-positive sera stained for IgM and IgG. Serum 1 shows multiple IgM-reacting bands, whereas serum 2 shows IgM reactions with only the 60- and 66-kDa bands. These two sera each had a barely perceptible band at <14 kDa after IgG reaction with this concentration of gel, but they were too faint to appear on the photo reproduction.

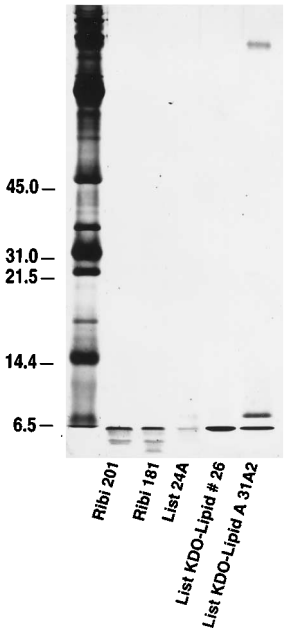

FIG. 3. Silver-stained 15% PAGE of (left to right) molecular weight standards, MPL Ribi lot 201, MPL Ribi lot 181, MPL List lot 24A, KDO-lipid A List lot 26, and KDO-lipid A List lot 31-A2. The more concentrated gel provides finer distinctions of the bands, which are consistent with dimers and trimers of lipid A. Sizes are shown in kilodaltons.

and diluted 1:10,000. Color was developed with *O*-phenylenediamine after a further five washes with buffer.

Competitive inhibition ELISA. Competitive inhibition ELISA was performed by adding either one of the MPL analogs or one of the antigens to tubes of serially diluted patient sera or synovial fluids of various known anti-MPL titers. All potential inhibitors were used at 50 μg/ml. These suspensions were incubated for 2 h at room temperature on a mechanical shaker before being transferred to microtiter plates and assayed by ELISA as described above. The percent inhibition was calculated as [1 − (optical density of the sample dilution plus antigen)/(optical density of the sample dilution alone)] × 100.

Since most sera had the highest optical density (O.D.) value in the uninhibited ELISA at 1:40, and since the first 10 sera studied were blocked most completely by MPL analogs at 1:20 or 1:40, most inhibition assays were performed at dilutions of only 1:10 and 1:40.

RESULTS

Electrophoretic and immunoblot searches for contaminants. The different lots of MPL used in these studies gave consistent quantitative results, although relatively lower absolute titers than those reported before (12). Two exceptions were notable. List lot 15 did not react with any sera when used in the ELISA but did block reactions of sera with Ribi lot 171 on ELISA plates and did produce a reactive line on immunoblots, and List lot 24A gave similar IgG titers with all sera tested, including sera from normal persons that did not react with other lots tested.

After electrophoresis with MPL or KDO-lipid A, 12.5% polyacrylamide gels stained with Coomassie blue showed faint, smudged bands at less than 14 kDa but no other stainable material. Silver staining of the 12.5% gels showed bands smaller than 14 kDa, and a band at 66 kDa was sometimes stained, but no other material appeared to be present (data not shown).

After transfer to nitrocellulose, bands at 60 and 66 kDa were detectable by India ink in all lots (Fig. 2), including the synthetic MPLs (not shown). Individual sera produced different patterns when stained for anti-IgM reactions (Fig. 2). IgG reactions were absent or limited. IgM reacted with the 60- and 66-kDa bands transferred from 12.5% gels in the lot of MPL (List 15) which had no reaction in ELISA. Both 14- and 66-kDa bands were detected by anti-IgM and anti-IgG in LA-16-PH, which had not been derived from a bacterial source but was reactive in the ELISA.

When 15% gels and lower-molecular-weight standards were used, the low-molecular-weight material resolved into several distinct bands at about 6.5 kDa and smaller. Each lot had a distinct pattern when silver stained (Fig. 3). The higher-molecular-weight material of the 12.5% gels was not seen in the 15% gels except in one lot of KDO-lipid A.

All sera from patients had IgG which reacted with an identical band on immunoblots (Fig. 4). This band was at just less than 6.5 kDa. A positive reaction in ELISA was always correlated with the presence of this band on immunoblots and vice versa.

Immunoblots of reactions with the aberrant lot 24A had bands identical to those of other lots when stained by patient sera but also sometimes had a band of slightly higher molecular weight (Fig. 4 and 5). In two of four immunoblots with normal sera, the lines appeared identical to those obtained with patient sera, but with the other two normal sera, the reaction was with the higher-molecular-weight line (Fig. 5).

Competitive inhibition with lipid A analogs. The results of the competitive inhibition experiments with the higher-molecular-weight analogs of MPL were expressed as the proportion of sera which had the O.D. reduced 50% or greater at a 1:40

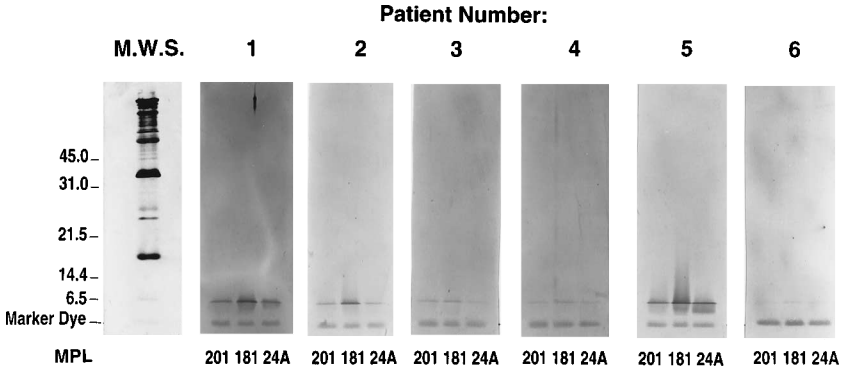

FIG. 4. Immunoblots obtained by 15% PAGE and developed with anti-IgG. Sera from six different pauciarticular patients were used against each of three different lots of MPL, the representative Ribi lots 181 and 201 and the anamalous lot 24A. In all cases, a single and apparently identical band is stained. Lane M.W.S., size standards (in kilodaltons).

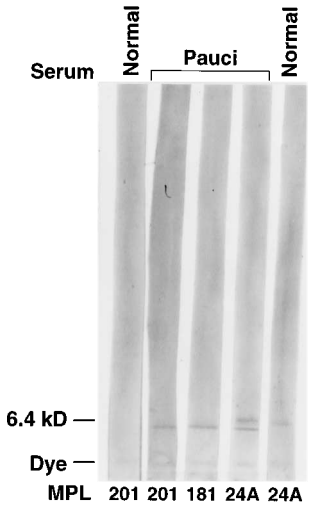

FIG. 5. Immunoblots obtained from 15% PAGE and developed with anti-IgG. The normal serum (both outside channels) does not react with lot 201 but does react with a higher-molecular-weight band in lot 24A. The pauciarticular juvenile arthritis patient's serum reacts with a single band in lots 201 and 181 but with both the same and the higher-molecular-weight band in lot 24A.

dilution in the presence of the analog and as the mean and standard deviations of percent blocking of sera at this dilution (Table 1). Strong inhibition occurred when MPL itself was used. KDO-lipid A inhibited the anti-MPL IgG reaction of only one of the six sera of pauci- and polyarticular origin and did not block anti-MPL IgM. MPL which had been treated with alkali to remove fatty acids inhibited more consistently than KDO-lipid A but less thoroughly than intact MPL. It did not inhibit the IgG reaction as well as the IgM reactions. The two synthetic MPL derivatives inhibited the serum and synovial fluid IgM from pauciarticular patients better than those from polyarticular patients, but they only inhibited the anti-MPL IgG in one serum and one synovial fluid sample. Three of the synovial fluid samples were matched with simultaneously obtained serum samples, and the results of all inhibition studies were essentially the same in the paired samples (data not shown).

The lower-molecular-weight compounds diglucosamine and monoglucosamine demonstrated optimal inhibition at the concentrations used when the sera were diluted to 1:20. Blocking of anti-MPL IgG was not striking but was demonstrated for two sera with GMDP and one serum with MDP. If we reduced our criteria to 25% blocking, four of six sera were inhibited by each of these small analogs.

Inhibition with biologic antigens. When we plotted full titration curves of experiments with biologic antigens of high and varied molecular weight, inhibition was obtained but at different titers with different sera. It was not possible to use a single dilution of serum for all of the antigens. For that reason, complete serum titrations were performed for each antigen with each serum. Examples are shown in Fig. 6. Inhibition was deemed to be present if at any point on the titration curve there was 50% or more reduction in O.D. in the presence of that antigen. The results are expressed in Table 2 as the ratio of the number of sera inhibited at any dilution to the number of sera tested. We found inhibition in five of six sera of anti-MPL by collagen types I and II. Cardiolipin blocked three of six sera. We did not find inhibition with proteoglycan, keratan sulfate, or DnaK heat shock protein from *E. coli.*

DISCUSSION

The most important finding was the discovery of a single common band of reactivity on immunoblots with IgG in all sera positive for IgG in the ELISAs. Other bands of materials were identified on PAGE and IgM immunoblots, but were not identified by IgG. It must be noted that we compared lipid with protein standards in the PAGE, so that exact molecular weights cannot be assigned.

Coomassie blue did not reveal any stainable contaminating material in PAGE gels. Silver stains of 12.5% PAGE gels did show unpredicted material of approximately 66 kDa in most lots of MPL. Immunoblots showed IgM reactivity at 66 and 60 kDa. The same reactivities were present in synthetic MPLs and were therefore not derived from the bacterial sources. We believe that these bands represent large polymers of MPL. The 15% gels stained with silver showed only low-molecular-weight bands of less than 6.5 kDa, consistent with dimers and trimers of MPL, the size of the IgG-reactive material.

The ability of the lot of MPL (List 15) which did not react in the ELISA to inhibit the reaction with a lot which did (Ribi 171) and its reaction in immunoblots imply that the relevant epitope was present on that batch of MPL. The differences in the reactions in the ELISA which caused this anomalous behavior, the variations in titers from batch to batch, and the high standard deviations in inhibition by similar analogs with different sera probably relate to a physical difference of the MPL, its oligomers, or bilayer forms when bound to the plastic com-

TABLE 1. Inhibition of anti-MPL ELISA of sera and synovial fluids by analogs of MPL

Juvenile arthritis type, sample,[a] and Ig class	No. of samples with 50% reduction in O.D./no. tested (mean % inhibition of all samples tested ± SD) with inhibiting analog:							
	MPL (Ribi 181)	MPL (List 15)	KDO-lipid A	Alkali-treated MPL	LA-16-pH	LA-15-pH	GMDP	MDP
Pauciarticular								
Serum IgG	8/10 (71 ± 31)	4/6 (58 ± 40)	1/6 (13 ± 31)	2/6 (26 ± 34)	1/6 (17 ± 41)	1/6 (12 ± 30)	2/6 (32 ± 23)	1/6 (18 ± 20)
S.F. IgG	3/4 (74 ± 28)	3/4 (67 ± 28)	—[b]	3/4 (58 ± 40)	1/4 (28 ± 31)	1/3 (20 ± 35)	—	—
Serum IgM	6/6 (97 ± 6)	6/6 (94 ± 7)	0/6 (0)	4/6 (51 ± 17)	6/6 (69 ± 17)	3/6 (49 ± 29)	—	—
S.F. IgM	4/4 (98 ± 2)	4/4 (98 ± 3)	—	3/4 (66 ± 46)	4/4 (86 ± 14)	3/3 (69 ± 19)	—	—
Polyarticular								
Serum IgG	5/6 (75 ± 39)	5/6 (70 ± 38)	1/6 (12 ± 27)	1/6 (29 ± 30)	0/6 (6 ± 9)	0/6 (2 ± 5)	—	—
S.F. IgG	1/2 (48 ± 54)	1/2 (43 ± 61)	—	0/2 (0)	1/2 (27 ± 38)	1/2 (30 ± 43)	—	—
Serum IgM	6/6 (99 ± 1)	6/6 (91 ± 13)	0/6 (0)	4/6 (66 ± 31)	2/6 (48 ± 31)	2/6 (41 ± 37)	—	—
S.F. IgM	2/2 (96 ± 4)	2/2 (91 ± 13)	—	1/2 (40 ± 23)	0/2 (22 ± 26)	1/2 (46 ± 11)	—	—

[a] S.F., synovial fluid.
[b] —, not studied.

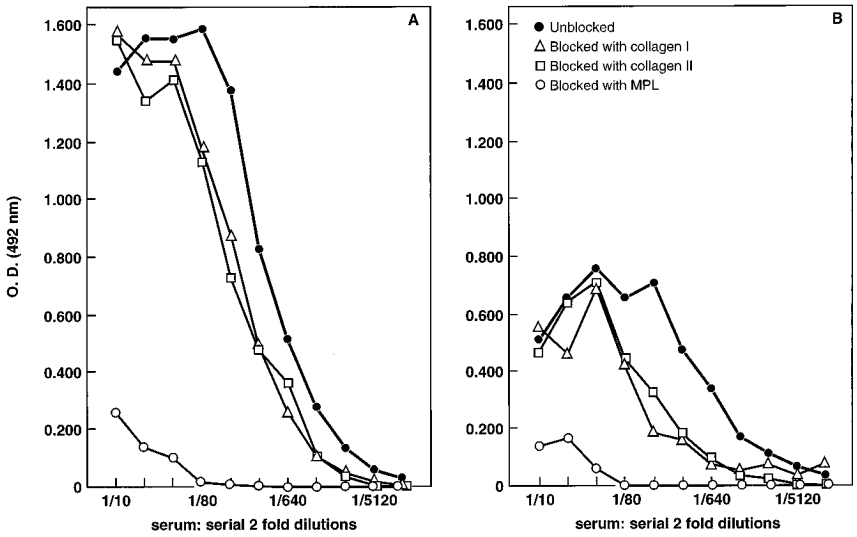

FIG. 6. Graphs showing the curves of O.D. plotted against dilutions of the representative sera unblocked or blocked by MPL, collagen type I, or collagen type II. Each serum produced a different curve and blocking pattern. Blocking was assumed to have occurred when the O.D. was depressed 50% at any one dilution, i.e., from 1:1,280 up in sample A and from 1:160 to 1:1,280 in sample B.

pared with their states in suspension rather than to a difference in antigenicity of the binding site per se. It is known that antisera can be very specific for minor variations in or very limited portions of the polar region of lipid A (8).

The behavior of List lot 24A, binding with IgG in all sera, remains unexplained. Nonspecific binding of LPS to aggregated IgG is known to occur (2), and we have found that heating sera to 56°C will increase titers. However, we were able to competitively block the reaction with lot 24A in immunoblots by using the more representative lot 201 as an inhibitor. The behavior of this lot was consistent in ELISA and immunoblot.

The data from the competitive inhibition of the anti-MPL reaction in the ELISA with different fractions of the MPL molecule support the prediction that the diglucosamine core is the major component of the immunologic binding site of MPL (1, 4, 8). KDO-lipid A, with the irrelevant saccharides covering the diglucosamine, is the least effective inhibitor. MPL which has lost fatty acids through alkali hydrolysis and the synthetic MPLs LA-16-pH and LA-15-pH, which share exposed diglucosamine cores but differ in fatty acids, are better inhibitors than KDO-lipid A. Moreover, diglucosamine or monoglucosamine on small peptides (GMDP or MDP, respectively) are capable of some inhibition of the reaction.

TABLE 2. Inhibition of anti-MPL IgG ELISAs of sera from children with pauciarticular juvenile arthritis by potential autoantigens

Potential autoantigen	No. of samples with 50% or greater inhibition of O.D. at any serum dilution/ no. tested
Fetal bovine cartilage collagen type I	5/6
Fetal bovine cartilage collagen type II	5/6
Proteoglycan	0/6
Keratan sulfate	0/6
Cardiolipin	3/6
DnaK heat shock protein	0/4

The inhibition of the anti-MPL reaction by the collagen preparations was unexpected. We had expected to find inhibition with proteoglycan or keratan sulfate, which contain saccharide chains as core structures. Cardiolipin was tested as a competitive inhibitor for this reaction primarily because of its recent clinical interest. It was at first surprising that it inhibited any sera, because the only structural similarity with MPL is the presence of a fatty acid and a small polar site; however, this reactivity has been confirmed with IgM monoclonal anti-KDO-lipid A developed for clinical use in gram-negative sepsis (1, 2). Indeed, IgM monoclonal antibodies specifically developed and purified against lipid A have been demonstrated to react with carbohydrates of a variety of cellular and macromolecular antoantigens (1). Bhat et al. (1) propose that anti-lipid A activity is part of the IgM "natural autoantibody" repertoire. The unique aspect of the antibody that we have been studying may be that it is IgG.

The cross-reactivity with cardiolipin is particularly interesting because studies in several laboratories have found that about 50% of sera from children with pauci- and polyarticular arthritis have anticardiolipin IgG activity (3, 15–17). Anticardiolipin activity in children with arthritis may represent cross-reacting anti-MPL antibody, since the latter is more common, but both reactivities may be secondary to some other, more significant reactivity (see below). The poor blocking of anti-MPL by KDO-lipid A is evidence that contaminating LPSs in the cardiolipin would not have caused false-positive inhibitory effects.

The fact that the anti-MPL cross-reacts with collagen is consistent with the possibility that this antibody is polyreactive and may have a biological effect because of the interaction with autoantigens. However, in our previous studies, anticollagen antibodies detected by ELISA did not correlate with complement activation (11). The correlation between C3 activation products and antibody titers has been true only of the anti-MPL IgG in our experience. Thus, the anti-MPL reaction is not just a cross-reaction of the usually measured anticollagen antibodies. However, it is still possible that the anti-MPL reaction is secondary to a primary reaction involving cartilage

and that what we are measuring is in fact a secondary phenomenon.

The ability of the agents studied to block the anti-MPL IgM was more complete than the ability to block the anti-MPL IgG. In addition, the IgG reactions varied more from serum to serum. This raises the possibility that anti-MPL IgG in these children is of different specificity than the more classically studied anti-MPL IgM.

This antibody remains of interest because of the correlations with complement activation (12), the increased concentration in synovial fluid (12), and the possible correlations with numbers of inflamed joints (10). The data presented in this paper indicate that the reaction that we are dealing with is not due to a contaminant of the MPL studied and that it is a reaction with the expected polar region on the MPL molecule. However, it also shows that there may be biological cross-reactivity between this antigen and antigens of significance in potential autoimmune reactions within joints.

ACKNOWLEDGMENTS

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